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In vitro screening assay for teratogens using growth inhibition of human embryonic cells

(cell proliferation/palate/human embryo/mesenchymal cell/metabolic activation)

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ABSTRACT **We have tested 35 teratogenic and 20 nonteratogenic chemicals or drugs in a short-term, *in vitro* assay that identifies teratogens by their ability to inhibit growth of an established line of human embryonic palatal mesenchymal cells. Only those chemicals that exhibited a dose-dependent inhibition of growth at concentrations less than 1 mM were classified as inhibitory. An Aroclor-induced rat liver S-9 system was effective in metabolizing cyclophosphamide to its teratogenic form in culture. We suggest that this assay, along with the complementary tumor cell-attachment assay of Braun *et al.* [Braun, A. G., Emerson, D. J. & Nichinson, B. B. (1979) *Nature (London)* 282, 507–509] may be useful as a short-term *in vitro* battery for assessment of the teratogenic potential in environmental agents and to prioritize those chemicals which merit further testing *in vivo*.**

The application of *in vitro* systems to teratogenicity testing has been of interest to teratologists and developmental biologists for many years (1, 2), with a dramatic increase in research in this area occurring after a workshop on this subject was held in 1981 (3). It has become quite evident that the current whole-animal test procedures for teratogenesis are inadequate to screen the large number of chemicals already present as well as new ones being introduced into the environment each year (4). Whole animal tests suffer from high cost, large number of animals used (mainly rodents and rabbits), and large amounts of chemical and time necessary for each full-scale determination.

The use of short-term *in vitro* screening systems could serve to establish priorities for the selection of chemicals that should be tested *in vivo*, could decrease the need for (but certainly not replace) whole animal tests, and ultimately could accelerate the time in which potential teratogens of human consequence can be evaluated. Various *in vitro* systems, which have been used to investigate normal embryogenesis, have been proposed, including cell, organ, and whole-embryo cultures derived from vertebrates as well as invertebrates (1–3). These systems reflect various embryonic events that occur during organogenesis, including cellular migration, interactions, proliferation, and differentiation; these events are known to be especially sensitive to teratogenic insult (4). It is highly unlikely that any one single *in vitro* system will suffice to reflect all of these events; instead a battery of complementary assays will be needed for teratogen screening.

Rapid cellular proliferation is essential for the early phases of organogenesis, and a number of chemicals and drugs, especially craniofacial teratogens, have been shown to interfere with this event in a specific manner (5). Several years ago, an established cell line (HEPM) was derived by using the mesenchymal cells of the secondary palate (roof of the mouth) from a day 55 human embryonic abortus (6). Subsequent studies of ours have examined the mechanism by which specific hormones and growth factors influence HEPM cell proliferation (7).

The purpose of the present study was to examine the potential for using teratogen-induced growth inhibition of the HEPM cells as a teratogen screening assay.

MATERIALS AND METHODS

Human embryonic palatal mesenchyme cells (HEPM) were originally established in culture by R. M. Pratt and the American Type Culture Collection (Rockville, MD) and designated as CRL 1486 (6). Passage 3 cells from the American Type Culture Collection were carried in our lab to passage 6–7, refrozen, and stored under liquid nitrogen in complete Dulbecco's modified Eagle's medium (DMEM) with 20% fetal calf serum and 10% dimethyl sulfoxide. Experiments in the present study were routinely performed with cells of passage 9 through 12. HEPM cells were plated in 35-mm Falcon tissue culture dishes at $4–5 \times 10^4$ cells per dish ($4.2–5.2 \times 10^3$ cells per cm^2) in 2 ml of DMEM (GIBCO 430-1600) (pH 7.4) containing glutamine (0.6 mg/ml), penicillin G (50 units/ml), streptomycin sulfate (50 μg/ml) (Sigma), and 10% fetal calf serum (GIBCO 200-6140, Hyclone lot 100397). Cultures were incubated in humidified air containing 7% CO_2, which maintained the pH at 7.3 ± 0.1. After 24 hr of culture, the medium was replaced (at 37°C) with or without the test chemical; the HEPM cells were maintained in culture for an additional 72 hr without a medium change. The cellular plating efficiency determined at 24 hr was >95%.

Test chemicals were dissolved at their solubility limit in medium, water, ethanol, or dimethyl sulfoxide. Ethanol and dimethyl sulfoxide final concentrations did not exceed 0.5% and 0.1%, respectively. A range-finding series of 4–5 concentration points was performed for initial runs to determine the upper concentration that almost completely inhibits cell growth without being toxic (loss of attachment or nonviable by the trypan blue exclusion test) and the lower concentration that only inhibits cell growth by 10–15%. The final IC_{50} (concentration that inhibits growth by 50%) concentration-finding run had at least 6 concentration points (mean of three to four dishes). At the termination of the experiment (total of 96 hr of culture), the cells were washed in calcium/magnesium-free phosphate-buffered saline and were detached in 0.05% trypsin/0.02% EDTA. Cell number was determined using a Coulter ZBI Counter with three readings for each dish. Net growth was calculated by subtracting the

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Abbreviations: HEPM cells, human embryonic palatal mesenchyme cells; MOT assay, mouse ovarian tumor cell-attachment assay.

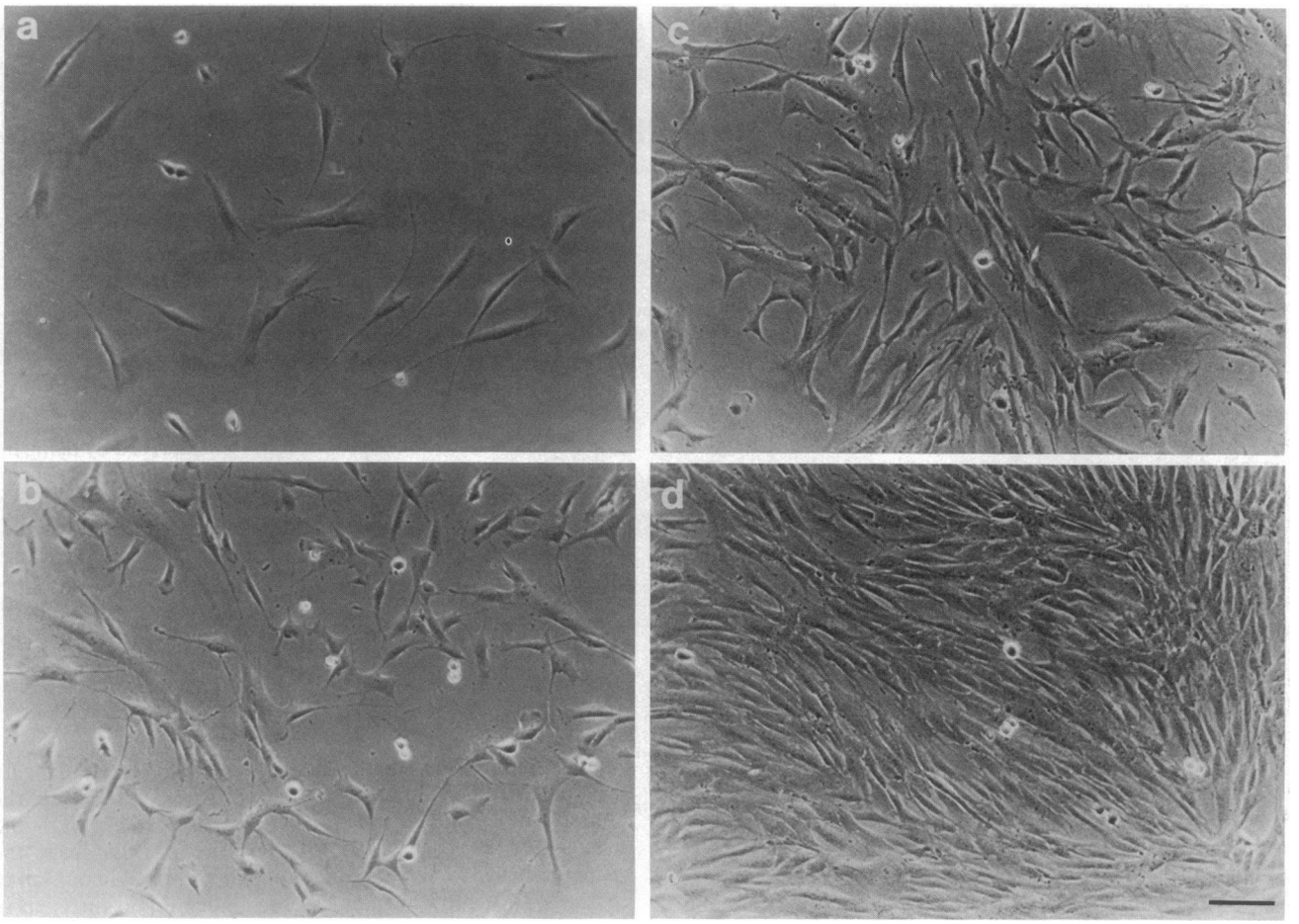

FIG. 1. Phase-contrast micrographs of HEPM cells (control) at 24 (*a*), 48 (*b*), 72 (*c*), and 96 (*d*) hr of culture. Cells were cultured as described in the text. (Bar = 100 μm.)

plating number at 24 hr from the final cell number. The resulting percentages were plotted on semilogarithmic graph paper against the test chemical concentration expressed as μg/ml or molarity; an IC_{50} for growth inhibition was then interpolated. The test chemicals were all obtained from Sigma except for the following ones: 13-*cis*-retinoic acid (National Toxicology Program, National Institute of Environmental Health Sciences), chlorcyclizine and meclizine (Beth Horigan, National Institute of Dental Research, Bethesda, MD), trypan blue (Matheson Coleman and Bell, Norwood, OH), Nembutal (Abbott), and colchicine (GIBCO).

Cyclophosphamide was chosen for metabolic activation studies because this chemical has been used in a number of studies either *in vivo* or in whole embryo culture (8, 9). Either Aroclor- or noninduced rat liver S-9 prepared in KCl and obtained from Litton Bionetics or Microbiological Associates was added directly to the HEPM cell cultures. Twenty-four hours after plating, the growth medium was removed, and S-9 and cofactors (10) in 0.5 ml of phosphate-buffered saline at pH 7.4 were added to fresh medium along with cyclophosphamide at 0.25–100 μg/ml to provide final concentrations as follows: 0.8 mg of S-9 protein per ml, 0.8 mM NADP, 10 mM glucose 6-phosphate, and 1.6 mM $MgCl_2$ in sodium phosphate buffer (20 mM) at pH 7.4. After 4 hr at 37°C, the reaction mixture was removed, cells were rinsed several times in

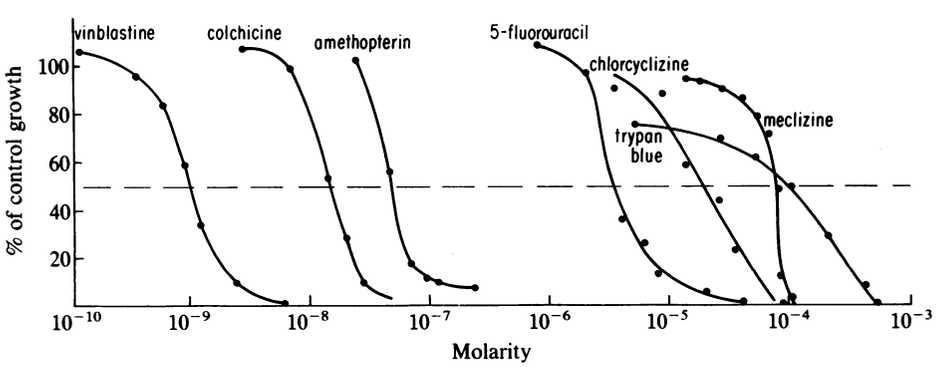

FIG. 2. Semilogarithmic plot of teratogens from Table 1 that were positive in the HEPM assay and negative in the MOT assay. The horizontal dashed line is the IC_{50}.

Table 1. Chemicals tested in the HEPM assay

	IC_{50}		
	M	μg/ml	M_r
Teratogens that are inhibitory *in vitro*			
Vinblastine*	1.1×10^{-9}	0.001	909.1
Vincristine sulfate	1.6×10^{-9}	0.0015	927.1
Colchicine*	1.5×10^{-8}	0.006	399.4
Amethopterin	5.3×10^{-8}	0.024	454.5
Cycloheximide	3.0×10^{-7}	0.085	281.3
5-Fluorouracil*	2.8×10^{-6}	0.37	130.1
6-Diazo-oxo-L-norleucine	3.2×10^{-6}	0.54	171.2
Arsenate	1.2×10^{-5}	2.3	185.9
6-Aminonicotinamide	1.5×10^{-5}	2.0	137.1
Diethylstilbestrol	1.8×10^{-5}	4.9	268.3
Chlorcyclizine*	2.3×10^{-5}	6.8	300.8
Dexamethasone	3.5×10^{-5}	14.0	392.5
Retinoic acid (*trans*)	6.8×10^{-5}	20	300.4
Dopamine	7.2×10^{-5}	11	153.2
Meclizine*	7.2×10^{-5}	28	391.0
Hydroxyurea	9.9×10^{-5}	7.5	76.1
Retinoic acid (13-*cis*)	1.0×10^{-4}	30	300.4
Trypan blue*	1.2×10^{-4}	115	960.8
Actinomycin D*	1.3×10^{-4}	163	1,255.5
L-Dopa	1.5×10^{-4}	30	197.2
Hydrocortisone	2.2×10^{-4}	80	362.5
Atropine sulfate	3.7×10^{-4}	250	676.8
Chloramphenicol	6.2×10^{-4}	200	323.1
Nonteratogens that are inhibitory *in vitro*			
Ouabain	8.8×10^{-9}	0.0064	728.6
Diphenhydramine	1.4×10^{-4}	40	291.8
Butylated hydroxyanisole	2.0×10^{-4}	36	180.2
2,4-Dinitrophenol	2.9×10^{-4}	53	184.1
Tween 80	3.1×10^{-4}	410	130.8
Isoniazid	5.0×10^{-4}	68	137.2
Phenol	8.3×10^{-4}	78	94.1
Acetaminophen	9.9×10^{-4}	150	151.2
Nonteratogens that are not inhibitory *in vitro*			
Metyrapone	1.2×10^{-3}	265	226.3
Ascorbic acid	1.7×10^{-3}	300	176.1
Cyclohexylamine	2.0×10^{-3}	200	99.2
Pyridoxine HCl	4.0×10^{-3}	830	205.6
Streptomycin sulfate	6.2×10^{-3}	10,000	1,619
Ammonium chloride	8.4×10^{-3}	450	53.5
Thiamine HCl	1.8×10^{-2}	830	205.6
Saccharin	2.6×10^{-2}	5,350	205.2
L-Lysine	2.7×10^{-2}	5,000	182.7
Cyclamic acid	3.2×10^{-2}	6,500	201.2
Sodium chloride	6.0×10^{-2}	3,500	58.4
L-Glutamine	6.6×10^{-2}	9,700	146.2
Teratogens that are not inhibitory *in vitro*			
Caffeine	1.2×10^{-3}	225	194.2
Nembutal	1.3×10^{-3}	320	248.3
Amaranth	1.5×10^{-3}	890	604.3
Theophylline*	2.0×10^{-3}	365	180.2
Nicotine*	3.7×10^{-3}	600	162.2
Acetazolamide	4.0×10^{-3}	900	222.2
Aspirin	4.2×10^{-3}	755	180.2
L-Phenylalanine	5.5×10^{-3}	910	165.2
Allopurinol*	7.7×10^{-3}	1,050	136.1
Penicillin G	8.1×10^{-3}	2,900	356.4
Urethane	3.3×10^{-2}	2,925	89.1
Sucrose	6.3×10^{-2}	21,500	342.3

The following chemicals, which are not inhibitory, are listed along with those amounts added to the culture medium above that present in DMEM: pyridoxine·HCl, thiamine·HCl, L-lysine, sodium chloride, L-glutamine, and L-phenylalanine.

*Teratogens that are false negatives in the MOT assay (12).

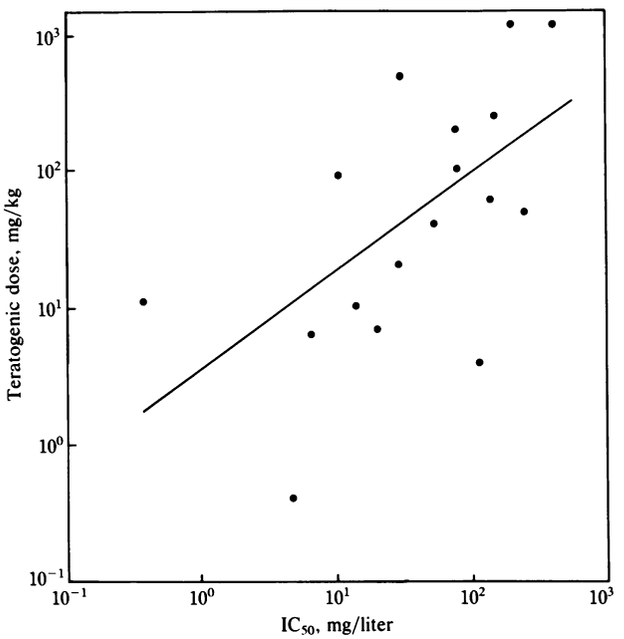

FIG. 3. Relationship between *in vitro* IC_{50} and the lowest reported (12) *in vivo* teratogenic dose. The data were taken from Table 1 and include 17 of the teratogens that are inhibitory *in vitro*. The line is based on the least-squares linear regression analysis.

phosphate-buffered saline, fresh growth medium was added, and the cultures were incubated for a further 72 hr without a medium change.

RESULTS

Our previous studies (11) have shown that, when plated at 4–5 × 10^4 cells per 35-mm dish, HEPM cells initiate at 18 hr a logarithmic increase in growth that lasts until day 4 (96 hr), after which the cells reach confluency. The fibroblast-like appearance of these untreated cells at various times during a typical 4-day test period is shown in Fig. 1. On day 4, the DNA and protein content per cell was 5.8 pg and 0.3 ng, respectively. This HEPM cell line has been passed 30 times (105 population doublings) in our laboratory, and a chromosomal analysis indicates an apparently normal diploid (46) human female karyotype (unpublished observations).

Most of the chemicals tested displayed a sigmoidal-shaped growth inhibition response (Fig. 2), and the IC_{50} varied from 1.1 nM to 66 mM (Table 1); 35 are teratogens and 20 are nonteratogens. It was not surprising to find that at a sufficiently high enough concentration, most chemicals produce some growth inhibition. However, this nonspecific growth inhibition at high concentrations does not appear to be related to mechanisms of animal or human teratogenicity; therefore, we have set 1 mM as the concentration below which specific (teratogenic) inhibition of growth occurs (see *Discussion*). By this criteria, 23 of the 35 teratogens had an IC_{50} ranging from 1.1 nM to 0.62 mM and were considered as inhibitory (Table 1). There appears to be some correlation (coefficient = 0.572) between the *in vitro* IC_{50} concentration and the lowest reported *in vivo* teratogenic dose (Fig. 3) for those teratogens that are inhibitory *in vitro* (Table 1). Of the 31 inhibitory chemicals or drugs, 23 (74%) were teratogenic in either animals or humans or in both. Among the 35 teratogens tested, 23 (66%) were inhibitory; 12 (60%) of the 20 nonteratogens were not inhibitory.

Although most of the known animal and human teratogens do not appear to require metabolic activation, there are some teratogen precursors (proteratogens; for instance, cyclophosphamide) that do require activation to their teratogenic form

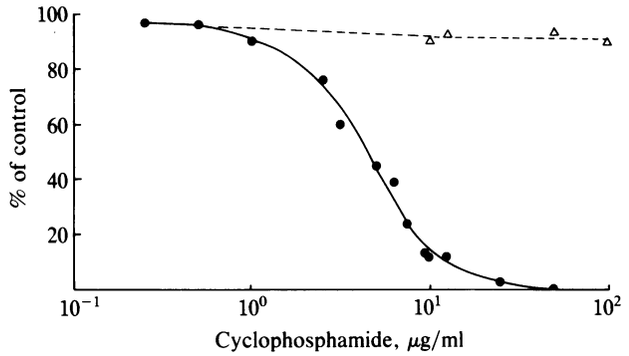

FIG. 4. Activation of cyclophosphamide in HEPM cell culture in the presence (●) or absence (△) of Aroclor-induced rat liver S-9 and cofactors as described in the text. The IC_{50} for CP was 4.5 μg/ml (17 μM).

(phosphoramide mustard). We have devised conditions in culture such that a 4-hr exposure to Aroclor-induced rat liver S-9 at 24 hr of culture results in a concentration-dependent activation of cyclophosphamide (IC_{50} = 4.5 μg/ml or 17 μM) (Fig. 4). Cyclophosphamide by itself up to 100 μg/ml did not inhibit growth, nor did cyclophosphamide with unactivated rat liver S-9 and cofactors.

DISCUSSION

The results from the present study indicate that the growth-inhibition assay using HEPM cells is a highly reproducible, simple, and inexpensive assay for determining teratogen-induced growth inhibition. It is quite clear that most chemicals we tested will produce growth inhibition if the concentration in the culture media is sufficiently high. These high concentrations (>1 mM) appear to have little relevance to animal or human teratogenesis and presumably represent nonspecific effects to the cell, in part due to the altered osmolarity. There is little information concerning the concentrations at which teratogens are present in the embryo under conditions that result in malformations. We have chosen 1 mM as the critical concentration below which growth inhibition is considered to reflect potential teratogenesis. A similar concentration range limit has been used in evaluating data from various chemicals in several different *in vitro* transformation assays including the mouse, rat, and hamster embryonic fibroblasts in culture (personal communication, Judson Spalding, National Toxicology Program, National Institute of Environmental Health Sciences).

The 55 chemicals chosen for testing in our study were mainly selected from the list of chemicals and drugs tested by Braun and co-workers (12–14) in their mouse ovarian tumor cell-attachment (MOT) assay. Most of the chemicals in Braun's list are animal teratogens that have been identified by a large data base; several of the chemicals (e.g., diethylstilbestrol and 13-*cis* retinoic acid) are also known human teratogens. We chose various representative chemicals from this list to test in the HEPM assay, including most of the false negatives in the MOT assay (Table 1); we suspected that these two assays, which measure different but essential events occurring during organogenesis, would be highly complementary. The results of the present study demonstrate that these two assays are highly complementary; the chemicals that are false negative in the MOT assay are positive in the HEPM assay. In both the HEPM and MOT assays, there is a reasonable correlation between the *in vitro* IC_{50} concentration and the lowest reported *in vivo* teratogenic dose for those teratogens that are inhibitory *in vitro*. The overall predictability in the combined MOT/HEPM assay is 90%, and the rate of false negatives is 3%; these are very important and desirable features because it is crucial that combined screening assays (batteries) do not miss potential human teratogens such as thalidomide. Although thalidomide is not inhibitory under any condition so far tested in the HEPM assay, it is inhibitory in the MOT assay in the presence of an S-9 activation system (10).

One of the advantages of the HEPM assay is that proteratogens, such as cyclophosphamide can be converted to their teratogenic form in a culture dish containing HEPM cells by using a limited (4 hr) exposure to an Aroclor-induced rat liver (metabolizing) S-9 system. Under these conditions, a concentration-dependent inhibition of growth is observed with an IC_{50} of 4.5 μg/ml (17 μM), which is nearly identical to the concentration in whole-rodent embryo culture that results in growth inhibition and malformations. For those activated proteratogens that may require continuous exposure in culture, the HEPM cells can be exposed for 4 hr to the S-9 activation system at 24-hr intervals during culture (unpublished observations).

Furthermore, the diploid HEPM cells can be maintained for at least 30 passages (105 population doublings) in culture. These human embryonic cells are much more sensitive to the growth-inhibitory effects of glucocorticoids (i.e., dexamethasone) *in vitro* as compared to adult skin fibroblasts (unpublished observations); in addition, these cells also may contain unique sensitivities peculiar to the human embryo. These cells may represent an undifferentiated neural-crest-like mesenchymal cell that is thought to be a likely target for many teratogens (5); therefore, we feel this cell line is unique and may be especially valuable for teratogen screening.

The MOT/HEPM battery has been tested with over 100 relevant chemicals to date (11–13). This does not imply that a final validation has been completed; however, the National Toxicology Program is currently in the process of validating this battery with the 44 chemicals suggested by Smith *et al.* (15).

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